Rac1 Mediates Dendrite Formation in Response to Melanocyte Stimulating Hormone and Ultraviolet Light in a Murine Melanoma Model

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Melanocytes are pigment producing cells that reside in the basal layer of the epidermis, and form multiple long dendritic processes that transport melanosomes from the melanocyte cell body to the dendritic tips, and then to keratinocytes. Dendrite formation requires actin polymerization in the newly forming dendrite, and dendrite formation in melanocytes is stimulated by hormones and ultraviolet light. The rho-subfamily of monomeric guanosine triphosphate-binding proteins is implicated in remodeling the cellular actin cytoskeleton, resulting in the formation of filopodia, lamellipodia, and stress fibers, as well as in oncogenesis and activation of the Jun/p38 mitogen activated kinase cascade. In this paper we show that rac1 induces the formation of dendrite-like structures when activated mutants are transiently expressed in B16F1 murine melanoma cells and in four human melanoma cell lines. Activated mutants of cdc42 and rhoA induced the formation of filopodia and stress fibers, respectively, in B16F1 cells, but not dendrites. A dominant negative inhibitor of rac1 abrogated the ability of α-melanocyte stimulating hormone, a peptide hormone known to stimulate melanocyte dendrite formation, and ultraviolet light, to induce dendrite formation in B16F1 cells, and α-melanocyte stimulating hormone and ultraviolet light stimulated the localization of rac1 to dendrite cell membranes. These results suggest that rac1 is an important signaling intermediate in dendrite formation in B16F1 cells, and that rac1 mediates the well-known ability of α-melanocyte stimulating hormone and ultraviolet light to induce dendrite formation. ***Key words: actin/GTP-binding protein/melanosome. J Invest Dermatol 111:243–250, 1998***

Melanocytes are neural crest derived cells that form dendrites, specialized cell processes that transport melanosomes to the tips of dendrites for transfer to keratinocytes, in response to hormones and ultraviolet (UV) light. Dendrites are critically important for efficient melanosome transfer, because one melanocyte makes contact with numerous keratinocytes in the epidermis through dendrite cell processes (Fitzpatrick *et al*, 1967). Although it is known that hormones, including α-melanocyte stimulating hormone (α-MSH), endothelin-1, and nerve growth factor, and UV light induce melanocyte dendrite formation, the molecular mediators are unknown (Hirobe, 1978; Friedmann and Gilchrest, 1987; Herlyn *et al*, 1988; Gordon *et al*, 1989; Yaar *et al*, 1991; Hirobe, 1992; Ivengar, 1994; Hara *et al*, 1995). Dendrites of vertebrate melanoma cells and melanocytes are composed of both actin filaments and microtubules, which are arranged parallel to the long axis of the dendrite, with microtubules in the center portion of the dendrite, and actin at the periphery (McGuire *et al*, 1972; Moellmann *et al*, 1973; Lacour *et al*, 1992). Confocal microscopy has shown that filamentous actin is particularly enriched immediately under the plasma membrane of dendritic tips and throughout the dendrite, compared with the cell body (Wu *et al*, 1997) and the initial process of dendrite formation in human melanocytes in response to UV radiation and to keratinocyte conditioned media has been shown to involve increased formation of microfilaments and actin polymerization (Jimbow *et al*, 1973; LaCour *et al*, 1992; Archambault *et al*, 1995). Similarly, dendrite extension in B16F1 cells in response to keratinocyte conditioned medium and in melanocytes in response to α-MSH has been shown to require actin assembly (Hirobe, 1978; LaCour *et al*, 1992).

Understanding the molecular basis of hormonally induced changes in cell shape has recently been advanced by the discovery that the rho-subfamily of guanosine triphosphate (GTP)-binding proteins orchestrate a variety of cellular shape changes, including lamellipodia, filopodia, and stress fiber formation through their ability to regulate actin assembly in response to growth factors (for review, see Bourne *et al*, 1991; Nobes and Hall, 1995a). Rac1, rhoA, and cdc42 are members of the rho subfamily of ras-related proteins, all bind and hydrolyze GTP and their activity is controlled by associated regulatory proteins. The active conformation is promoted by guanine nucleotide exchange factors, which promote the exchange of guanosine diphosphate (GDP) for GTP, and the inactive conformation is promoted by GTPase-activating proteins and GDP dissociation inhibitors (GDI). In addition to activating GTP hydrolysis, some GTPase-activating proteins, such as n-chaemerin, may also act as effector molecules in cytoskeletal reorganization (Kozma *et al*, 1996). RhoA stimulates stress fiber formation in response to lysophosphatidic acid, platelet derived growth factor, fetal calf serum, bombesin, and epidermal growth factor, and rhoA dependent stress fiber formation appears to depend upon tyrosine phosphorylation (Ridley and Hall, 1992, 1994; Nobes and Hall, 1995b; Flinn and Ridley, 1996). Rac1 and cdc42 have been shown to induce membrane

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Abbreviations: GDI, GDP dissociation inhibitors; MSH, melanocyte stimulating hormone; NDP-MSH, (Nle^4-D-Phe^7)-α-MSH.

ruffling and lamellipodia formation (rac1) and filopodia formation (cdc42), respectively, when active mutants are microinjected into cells (Nobes and Hall, 1995b). Constitutively active rac1 mutants injected into Swiss 3T3 cells induce the formation of focal complexes containing vinculin, focal adhesion kinase, and paxillin at the leading edge of newly formed lamellipodia (Nobes and Hall, 1995b). Rac1 has been shown to mediate the effects of a wide variety of hormones on membrane ruffling, as demonstrated by experiments in which dominant negative inhibitors of rac1, when microinjected into Swiss 3T3 cells, inhibited the ability of platelet derived growth factor, phorbol 12-Myristate 13-acetate, epidermal growth factor, bombesin, and insulin to induce membrane ruffling (Ridley *et al*, 1992). Cdc42 was originally isolated as a yeast mutant involved in defective cell budding and yeast polarity (Adams *et al*, 1990; Johnson and Pringle, 1990); its human homolog induces the formation of filopodia when active mutants are microinjected into cells (Nobes and Hall, 1995b) and cdc42 mediates bradykinin-induced formation of peripheral actin microspikes in Swiss 3T3 cells (Kozma *et al*, 1995).

α-MSH is a member of the melanotropic hormones, a family of structurally related peptides derived from one precursor protein, proopiomelanocortin (POMC). There are five different melanocortin receptors (MC1–MC5), a subfamily of G protein-coupled receptors with seven transmembrane domains (Mountjoy *et al*, 1992; Cone *et al*, 1993). The melanocortin receptors differ in their relative affinities for the various melanotropic peptides and melanocytes and melanoma cells express the MC1 receptor, which has a high affinity for α-MSH and its potent synthetic analog (Nle^4-D-Phe^7)-α-MSH (NDP-MSH). Binding of these peptide hormones to their cognate MC receptors results in activation of protein kinase A through activation of adenylate cyclase and increased production of cAMP, with subsequent mitogenesis and melanogenesis (Mac Neil *et al*, 1981; Hill *et al*, 1989; Hunt *et al*, 1994; Mountjoy, 1992; Abdel-Malek *et al*, 1995; Swope *et al*, 1995; Suzuki *et al*, 1996; for review see Hunt, 1995). Melanocyte stimulating hormone, as well as agents that increase cAMP levels, such as isobutylmethylxanthine and dibutyryl cAMP, have been reported to induce dendricity in melanocytes and melanoma cells (Hirobe, 1992; Hunt *et al*, 1994; Abdel-Malek *et al*, 1995). Another powerful stimulant of melanocyte dendricity is UV light. Within 24 h of irradiation of cocultures of normal human melanocytes and keratinocytes, melanocytes form long branching dendritic processes, primarily through the upregulation of endothelin-1 production by keratinocytes (Imokawa *et al*, 1995), which has been shown to be a powerful inducer of melanocyte dendrite formation (Hara *et al*, 1995). Melanocyte dendricity can also be induced by UV light in the absence of keratinocytes (Friedmann and Gilchrest, 1987), although the mechanisms are unknown.

We have used B16F1 murine melanoma cells as a model system to study the role of the rho subfamily of GTP-binding proteins in dendrite formation in response to the α-MSH analog NDP-MSH, and UV light. We show that activated rac1 proteins induce dendrite formation in B16F1 cells, and that a dominant negative inhibitor of rac1 abrogates the ability of NDP-MSH and UV light to induced dendrite formation in B16F1 cells. Further, we show that activated rac1 induces the formation of dendrites in four human melanoma cell lines. These results suggest that rac1 is a primary signaling intermediate for dendrite formation in melanocytic cells.

MATERIALS AND METHODS

Cells and cell culture The B16F1 mouse melanoma cell line was obtained from American Type Culture Collection (Rockville, MD). The cells were checked periodically for contamination with mycoplasma by staining with bisBenzimide Hoechst trichloride (Sigma, St. Louis, MO). B16F1 cells were maintained in Dulbecco's minimal essential media (Gibco BRL, Gaithersberg, MD) supplemented with 2% fetal calf serum (Gibco BRL). The A375P and A375M human melanoma cell lines were a gift of Dr. Ian Hart (Imperial Cancer Research Fund, London, U.K.) and were maintained in minimal essential medium (Gibco BRL) supplemented with 10% fetal calf serum and have been described previously (Marshall *et al*, 1991). The WM-793 and WM-98–1 human primary melanoma cell lines were a generous gift of Dr. Meenhard Herlyn (The Wistar Institute of Anatomy and Biology, Philadelphia, PA), and have been described previously (Albelda *et al*, 1990; Easty *et al*, 1995). They were maintained in MCDB-153/L-15 media (4/1; Gibco BRL) supplemented with 10% fetal calf serum.

Antibodies and reagents Rabbit polyclonal antibodies to murine tyrosinase-related protein (clone αPEP1) were a generous gift of Dr. Vincent Hearing (National Institutes of Health, Bethesda, MD) and have been previously described (Jimenez *et al*, 1988); polyclonal antibodies to myosin V (clone DIL2) raised against a portion of the myosin V heavy chain (residues 910–1106) were a generous gift of Dr. John Hammer (National Institutes of Health) and have been described previously (Wu *et al*, 1997). Monoclonal antibodies to the c-myc epitope (9E10) were purchased from Sigma; polyclonal antibodies to rac1 and control peptides for preabsorption of the antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate-conjugated goat anti-mouse and anti-rabbit antibodies, phycoerythrin conjugated goat anti-mouse antibodies, and normal goat serum were purchased from Sigma; NDP-MSH and dibutyryl cAMP were purchased from Sigma. NDP-MSH was used because it is more potent and more stable than α-MSH (Sawyer *et al*, 1982). Rhodamine phalloidin was purchased from Molecular Probes (Eugene, OR).

Immunofluorescence staining Cells on coverslips were fixed in 4% formalin for 10 min at room temperature. Cells were permeabilized by incubation in 0.5% Triton-X 100 in phosphate buffered saline (PBS) for 15 min at room temperature. Non-specific binding of antibody was blocked by incubation of the coverslips in 10% normal goat serum for 10 min followed by incubation with the primary antibody for 1 h at room temperature. After several washes in PBS, secondary antibodies were applied for 30 min at room temperature. For staining of filamentous actin, rhodamine phalloidin (1/40 in PBS) was added along with the secondary antibody. Coverslips were examined using a confocal microscope from Biorad, or with a Nikon immunofluorescence microscope.

Irradiation of cells The source of UV irradiation was a 1000 W Oriel Xenon Arc Solar Simulator with UVC WG320 and Visble cutoff filter UG11, beam turner, and cooling fan. This light source emits electromagnetic radiation from 295 nm (UV-B) to 400 nm and produces 3.75×10^{-3} W per cm^2. The output was monitored by an IL1700 radiometer and SED400 sensor probe purchased from International Light (Newburyport, MA). The desired dose of UV light (4.5 J per cm^2) was obtained by irradiating the cells for 20 min. During UV irradiation medium was replaced with warm PBS containing Ca^{2+} and Mg^{2+}. The PBS was then replaced with medium (Dulbecco's minimal essential media + 2% fetal calf serum).

Expression vectors and transfections All transient transfections were generated by transfection of 1×10^5 cells on glass coverslips with 2 μg of each pcEXV-3 eukaryotic expression vectors containing either V12rac1, N17rac1, V12cdc42, N17cdc42, V14rhoA, or A35rac1 engineered such that the protein was tagged with the c-myc epitope. All vectors were kindly provided by Dr. A. Hall (University College, London, U.K.), and are fully described in Ridley *et al* (1992). The expressed proteins of V12rac1, V12cdc42, and V14rhoA are consitutively active; the expressed proteins of N17rac1 and N17cdc42 function as dominant negative inhibitors; the expressed protein of A35rac1 is inactive. For transfections, cells were placed in OPTI-MEM(R) (Gibco BRL) and incubated with vectors and 10 μl lipofectamine (Gibco BRL) overnight at 37°C. Cells were then fixed and transfected cells were visualized by detection of the c-myc epitope with 9E10 antibodies followed by immunofluorescence microscopy as described above.

RESULTS

Actin and melanosomes co-localize in NDP-MSH induced dendrites To determine if treatment of B16F1 cells with NDP-MSH induced the co-localization of actin and melanosomes in dendrites, B16F1 cells were treated with NDP-MSH (10^{-8} M) for 1 h or 24 h and dual localization of melanosomes and the actin cytoskeleton was performed. Co-localization of actin and melanosomes was assessed by examination of stained coverslips by confocal microscopy (**Fig 1*a–c***). Melanosomes were located in the perinuclear area and throughout the cytoplasm in untreated cells (**Fig 1*a***) and some, but not all melanosomes, co-localized with filamentous actin in the cell cytoplasm as determined by confocal microscopy. Filamentous actin was present at the periphery of the cell, but melanosomes were generally absent from the cell periphery. After 1 h of treatment with NDP-MSH, small dendritic processes appeared in treated cells, and melanosomes were present at the tips of dendrites and co-localized with actin filaments (**Fig 2*b***). After 24 h of treatment with NDP-MSH, long branching

dendrites had formed and melanosomes were present at the dendritic tips and along the length of the dendrite (**Fig 2*c***). Dibutyryl cAMP (10^{-5} M for 1 and 24 h) also induced the formation of actin containing dendrites in B16F1 cells in which actin and melanosomes co-localized (data not shown).

Activated rac1 induces the formation of dendrite-like processes in B16F1 cells To determine if expression of V12rac1 would induce dendrites in B16F1 cells, cells were transfected with plasmids expressing the constitutively active mutant forms of the rac1 protein (V12rac1) and the morphology and actin cytoskeleton of transfected cells were examined by immunofluorescence microscopy. Cells were also transiently transfected with plasmids expressing constitutively active cdc42 protein (V12cdc42) and with plasmids expressing constitutively active rhoA protein (V14rhoA). Controls consisted of cells transfected with an inactive rac1 protein (A35rac1). Transfected cells were visualized by staining with antibodies to the c-myc epitope and the cytoskeleton was visualized by staining with rhodamine phalloidin (**Figs 2**, **3**), and each experiment was performed at least three separate times. Cells transfected with V12rac1 expression vectors exhibited a complex branching dendritic morphology (**Fig 2*a–f***; c-myc epitope) and the dendritic processes were composed of actin (**Fig 2*g***, rhodamine phalloidin stain of cell pictured in **Fig 2*f***). A low power photomicrograph of a field of V12rac1-transfected cells shows several transfected cells that are highly dendritic (**Fig 2*a***, ***b***); the arrowheads highlight nontransfected cells that are round or polygonal. In general, virtually all transfected cells exhibited a dendritic phenotype compared with nontransfected cells and with cells transfected with the A35rac1 plasmid. Even 3 d after transfection, V12rac1 expressing cells maintained their dendritic phenotype (not shown). Some V12rac1-expressing cells contained intracytoplasmic vesicles, which have previously been shown to represent hyperpinocytosis of the plasma membrane, seen in cells with prominent membrane ruffling (Ridley *et al*, 1992; not shown). Cells transfected with V12cdc42 showed numerous fine filopodia (**Fig 3*a***, ***b***) but did not display branching dendritic processes as seen in V12rac1 transfected cells. Filopodia can be seen in a V12cdc42 transfected cell (**Fig 3*a***; c-myc epitope) and the filopodia are composed of actin filaments (**Fig 3*b***, rhodamine phalloidin stain of cell pictured in **Fig 3*a***). Cells transfected with V14rhoA vectors were flattened and somewhat contracted compared with nontransfected cells (**Fig 3*c***; c-myc epitope), and transfected cells exhibited numerous closely packed actin stress fibers (**Fig 3*d***, rhodamine phalloidin stain of the same cell). Cells transfected with A35rac1 vectors (**Fig 3*e***; immunofluorescence staining for c-myc epitope) were round or polygonal, lacked dendrites, and had sparse actin fibers (**Fig 3*f***; rhodamine phalloidin stain of the same cell) similar to the morphology of untransfected B16F1 cells.

To determine if rac1 would induce the formation of dendrite-like processes in human cell lines, we transfected two human melanoma cell lines (A375P, A375M) with V12rac1, V12cdc42, V14rhoA, and A35rac1 vectors and stained slides were examined by immunofluorescence microscopy. In addition, two other human melanoma cell lines (WM-793, WM-98–1) were transfected with either V12rac1 or A35rac1. Each of these cell lines are round or polygonal and grow in small groups in culture, similar to B16F1 cells. Each of these cell lines exhibited a dendritic morphology following transfection with V12rac1, similar to what was seen in the B16F1 cells. **Figure 4** shows the morphology of WM-98–1 and WM-793 cells [parts (*a*) and (*b*), respectively], following transfection with V12rac1. Both cell lines displayed a markedly dendritic phenotype, in contrast with cells expressing the inactive rac1 protein, A35rac1 [(*c*), WM-98–1 cell expressing A35rac1]. **Figure 4(*d*)** shows an A375P cell expressing V12rac1 detected with antibodies to the c-myc epitope; **Fig 4(*e*)** shows the same cell visualized with a filter to detect rhodamine phalloidin. V12rac1 induced the formation of short dendrite-like structures in A375P cells composed of actin filaments. Cells transfected with V12cdc42 exhibited numerous filopodia (not shown). V14rhoA transfected A375P cells were flattened (**Fig 4*f***; c-myc epitope) and displayed increased closely packed actin stress fibers (**Fig 4*g***; rhodamine phalloidin stain of the same cell), but no evidence of dendrite formation. Results for the A375M cell line were similar and are not shown.

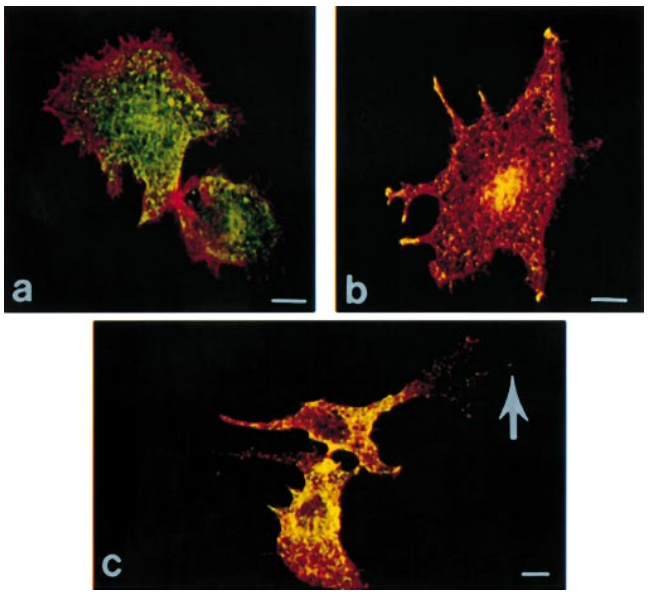

Figure 1. Melanosomes and actin co-localize in dendrites in B16F1 cells treated with NDP-MSH. B16F1 cells were grown in Dulbecco's minimal essential media + 2% fetal calf serum (*a*) or in the presence of NDP-MSH (10^{-8} M) for 1 h (*b*) or 24 h (*c*) and melanosomes were localized by staining with antibodies to tyrosinase related protein (αPEP1) and detected with fluorescein isothiocyanate-conjugated secondary antibodies. The actin cytoskeleton was visualized by staining with rhodamine phalloidin. The cells were then examined by confocal microscopy for co-localization of actin and melanosomes, indicated by a yellow color from an overlay of green and red. Untreated cells are round and lack dendrites (*a*). Melanosomes are present in a perinuclear distribution and throughout the cytoplasm. Some, but not all melanosomes co-localize with actin in the cytoplasm of untreated cells. Cells treated with NDP-MSH for 1 h show dendrite formation and melanosomes, present at the tips of dendrites, co-localize with actin (*b*). After 24 h of treatment with NDP-MSH, B16F1 cells have formed long branching dendrites and melanosomes in the cell body and in the dendritic processes co-localize with actin (*c*; *arrow*). *Scale bars*: (*a*, *b*) 5 μm, (*c*) 25 μm.

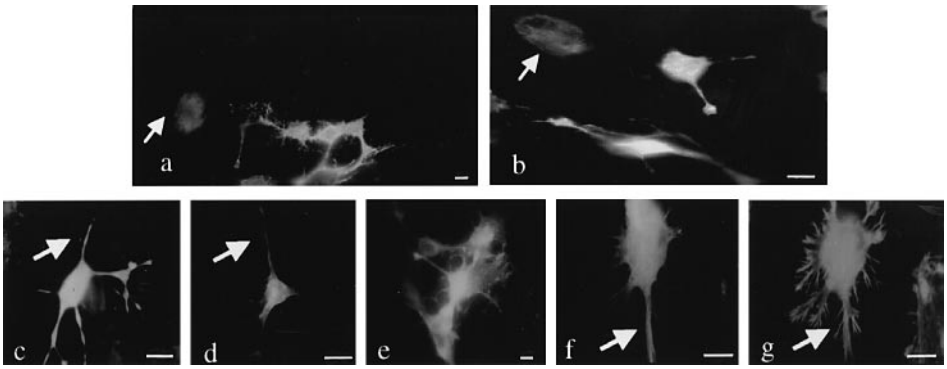

Figure 2. V12rac1 induces the formation of actin-containing dendritic processes in B16F1 cells. B16F1 cells transiently transfected with V12rac1 were detected by expression of the c-myc epitope (*a–f*). Low power photomicrographs (*a*, *b*; *scale bar*, 60 μm) show several transfected cells in the field that exhibit dendrites. In comparison, nontransfected cells (*arrows*) are round and nondendritic. Parts (*c*)–(*f*) show the V12rac1 transfected cells under higher power (*scale bar*, 20 μm). Cells contain thin, branching dendritic structures induced by expression of the V12rac1 protein (*arrows*). The dendrites were composed of actin, as shown by rhodamine phalloidin staining (*g*).

Rac1-induced dendrites contain melanosomes We next determined if the dendrite-like structures induced in V12rac1 expressing cells contained melanosomes. B16F1 cells were transiently transfected with V12rac1 vectors, and double labeling was performed for detection of the c-myc epitope and myosin V, and slides were examined by immunofluorescence microscopy. Myosin V co-localizes with the melanosome specific protein tyrosinase related protein-1 and actin filaments (Wu *et al*, 1997), thus the presence of myosin V in dendrite-like extensions in V12rac1 transfectants would suggest functional coupling between melanosomes and actin. **Figure 5**(***a***) shows a V12rac1 transfected cell, identified by staining with antibodies to the c-myc epitope and detected with phycoerythrin conjugated secondary antibodies; the same cell stained with antibodies to myosin V and detected with fluorescein isothiocyanate-conjugated secondary antibodies is shown in **Fig 5**(***b***). A long thin dendritic process extending from the cell body in a V12rac1-transfected cell is seen and examination of the distal portion of the dendrite reveals myosin V staining, indicating the presence of melanosomes in the dendrite. Untransfected B16F1 cells stained with antibodies to myosin V (**Fig 5*c***) demonstrate the expected location of myosin V in the perinuclear area of nondendritic B16F1 cells. A V12cdc42 transfected cell (**Fig 5*d***; c-myc epitope) exhibits a thin filopodia-like structure that does not contain melanosomes, as shown by double labeling with anti-myosin V antibodies (**Fig 5*e***; myosin V), indicating that these structures do not function as dendrites. **Figure 5**(***f***) shows normal rabbit serum as a negative control for myosin V staining in a nontransfected B16F1 cell.

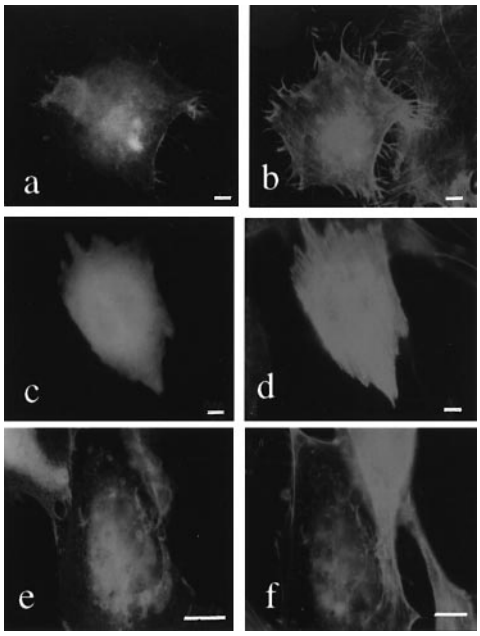

Figure 3. V12cdc42 and V14rhoA induce filopodia and stress fibers, respectively, in B16F1 cells. B16F1 cells transiently transfected with V12cdc42 (*a*, *b*) and V14rhoA (*c*, *d*) or A35rac1 (*e*, *f*) were detected by staining with antibodies to the c-myc epitope (*a*, *c*, *e*), and their cytoskeleton was visualized by the addition of rhodamine phalloidin with the secondary antibody (*b*, *d*, *f*). Cdc42-expressing cells exhibited fine filopodia (*a*, *b*), whereas V14rhoA expressing cells (*c*) were flattened and contained numerous dense and closely packed actin stress fibers (*d*). Cells expressing the inactive rac1 protein A35rac1 resembled nontransfected cells, and displayed a polygonal morphology (*e*) with a sparse actin cytoskeleton (*f*). *Scale bars*: 5 μm.

A dominant negative inhibitor of rac1 blocks the ability of MSH and UV light to induce dendrite formation in B16F1 cells For analysis of the role of rac1 in α-MSH induced dendrite formation, B16F1 cells were transiently transfected with a pcEXC eukaryotic expression vector containing a dominant negative inhibitor of rac1, N17rac1. Controls consisted of cells transfected with a dominant negative inhibitor of cdc42 (N17cdc42) or with A35rac1 expression vectors. Cells were then treated with NDP-MSH (10^{-8}–10^{-11} M) for 1–3 d following transfection and were stained with antibodies to c-myc to detect transfected cells and with rhodamine phalloidin (**Fig 6*a–f***). For analysis of the effect of rac1 on UV light induced dendrite formation, B16F1 cells were transfected with either N17rac1, N17cdc42, or A35rac1 overnight, and the next day cells were placed in sterile PBS and were irradiated with a single dose of UV light from a solar simulator at a dose of 4 J per cm^2 (shown to induce dendricity in B16F1 cells without cytotoxicity in preliminary experiments). Medium was replaced and 24 h later cells were stained with antibodies to the c-myc epitope to identify transfected cells, and with rhodamine phalloidin to visualize the actin cytoskeleton (**Fig 7*a–d***). Slides were examined by immunofluorescence microscopy.

N17rac1 completely inhibited the ability of NDP-MSH to induce dendrite formation at all time points and dosages of NDP-MSH; shown

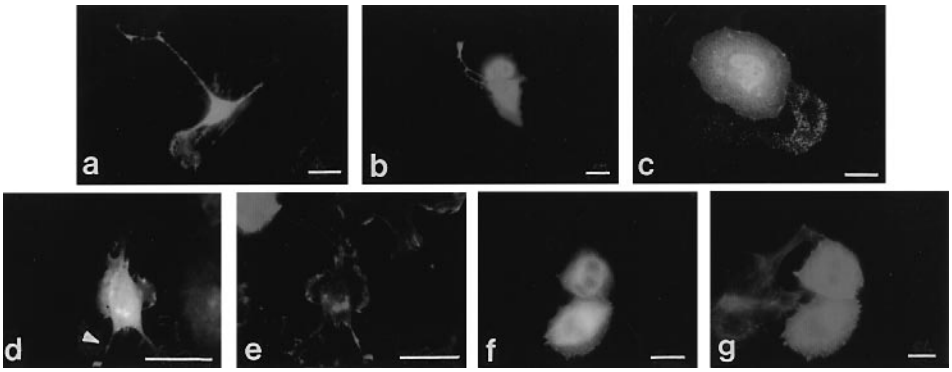

Figure 4. Human melanoma cell lines transfected with V12rac1 are dendritic. To determine if V12rac1 would induce the formation of dendrites in human melanoma cell lines, the WM-98–1, WM-793, A375P, and A375M melanoma cell lines were transfected with V12rac1 or A35rac1 and their morphology was visualized by staining with antibodies to the c-myc epitope tag. In addition, the A375M and A375P cell lines were transfected with V14rhoA. V12rac1 induced the formation of dendrites in all four human melanoma cell lines; shown here are WM-98–1 (*a*), WM-793 (*b*), and A375P (*d*) V12rac1-transfected cells identified by staining for the c-myc epitope. Transfected cells display long thin dendritic structures, which are composed of actin (*e*; rhodamine phalloidin stain of V12rac1-transfected A375P cell). Expression of the inactive A35rac1 protein had no effect on the morphology of the cells; shown in (*c*) is a WM-98–1 cell transfected with A35rac1 that is round and nondendritic. Transfection of the A375P cell line with V14rhoA induced a contracted and flattened phenotype (*f*, c-myc epitope) and cells contained dense stress fibers (*g*; rhodamine phalloidin stain). Results for the A375M cell line are similar and are not shown. *Scale bars*: (*a–c*, *f*, *g*) 10 μm, (*d*, *e*) 40 μm.

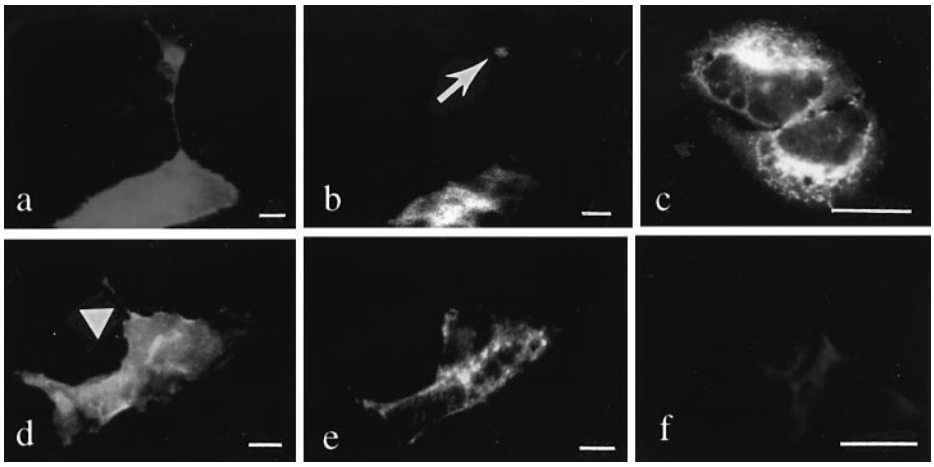

Figure 5. Dendrites induced by V12rac1 contain melanosomes. B16F1 cells were transiently transfected with V12rac1 (*a*, *b*) or V12cdc42 (*d*, *e*) and double immunofluorescence for detection of c-myc epitope (*a*, *d*) and myosin V (*b*, *e*) was performed. Negative controls for myosin V staining (normal rabbit serum) are shown in (*f*); a nontransfected cell stained for myosin V is shown in (*c*). V12rac1 expressing cells (*a*; c-myc epitope) exhibited thin dendritic cell processes and myosin V staining was seen (*arrow*) at the tip of the cell processes, indicative of the presence of melanosomes (*b*). Nontransfected cells (*c*; myosin V staining) showed the expected presence of melanosomes in the peri-nuclear (presumed Golgi) area. In contrast, cells transfected with V12cdc42, which induce the formation of short filopodia (*d*; *arrowhead*, c-myc epitope) did not contain melanosomes in the cell process (*e*; myosin V staining). *Scale bars*: 20 μm.

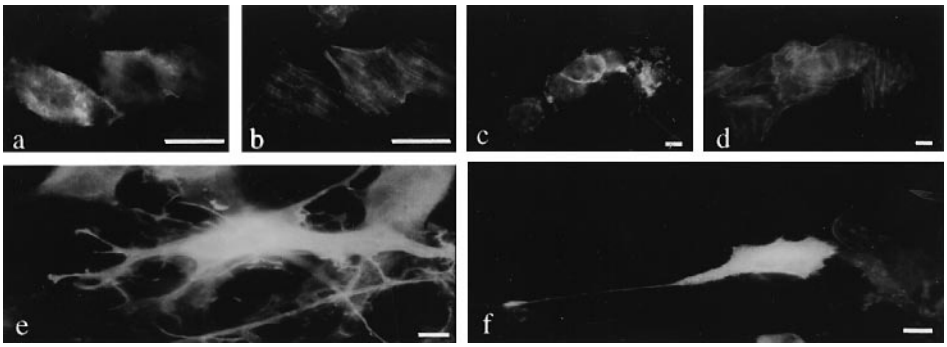

Figure 6. A dominant negative inhibitor of rac1 (N17rac1) abrogates the ability of NDP-MSH to induce dendrites in B16F1 cells. B16F1 cells were transiently transfected with N17rac1 (*a–d*), N17cdc42 (*e*), or A35rac1 (*f*) overnight and cells were then treated with NDP-MSH (10^{-8} M) for 24 h. Transfected cells were detected by staining with antibodies to the c-myc epitope (*a*, *c*, *e*, *f*); the actin cytoskeleton was detected by staining with rhodamine phalloidin (*b*, *d*). Cells expressing N17rac1 (*a*, *c*; c-myc epitope) were round or polygonal and did not contain dendrites. The actin cytoskeleton was sparse (*b*, *d*), similar to nontransfected cells. In contrast, cells expressing N17cdc42 or A35rac1 (*e* and *f*, respectively; c-myc epitope) formed branching dendritic structures in response to NDP-MSH. *Scale bars*: 10 μm.

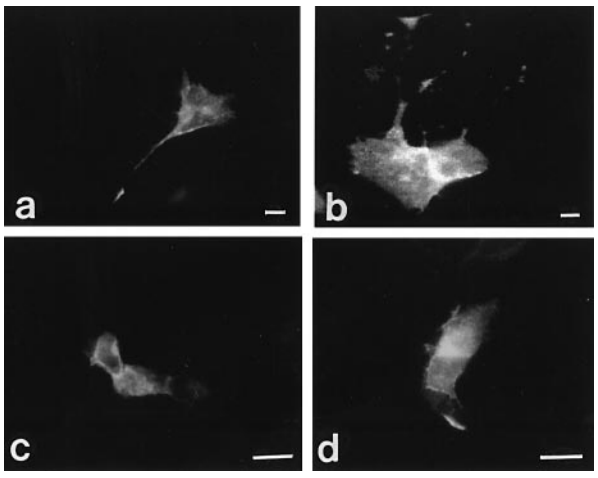

Figure 7. A dominant negative inhibitor of rac1 abrogates the ability of UV light to induce dendrites in B16F1 cells. B16F1 cells were transiently transfected with N17cdc42 (*a*), A35rac1 (*b*), or N17rac1 (*c*, *d*) overnight and cells were then irradiated with a single dose of UV light from a solar simulator (4.0 J per cm^2). Twenty-four hours later transfected cells were detected by immunofluorescence staining for the c-myc epitope tag (*a–d*). Cells expressing N17cdc42 (*a*) and A35rac1 (*b*) responded to UV light with the formation of dendrites; in contrast, cells expressing N17rac1 (*c*, *d*) failed to form dendrites in response to UV light. The actin cytoskeleton of these cells was sparse and did not contain stress fibers (not shown). *Scale bars*: 15 μm.

in **Fig 6** are results from 24 h of NDP-MSH treatment at 10^{-8} M. B16F1 cells transfected with N17cdc42 and A35rac1 (**Fig 6*e*, *f***, respectively; c-myc epitope) responded to NDP-MSH with the formation of branching dendritic cell extensions. In contrast, cells transfected with N17rac1 were flattened and round (**Fig 6*a*, *c***; c-myc epitope) and showed a sparse cytoskeleton as demonstrated by rhodamine phalloidin staining (**Fig 6*b*, *d***). N17rac1 also abolished the ability of UV light to induce dendrites in B16F1 cells (**Fig 7*a–d***). B16F1 cells transfected with N17cdc42 or A35rac1 (**Fig 7*a*, *b***, respectively; c-myc epitope) responded to UV light with the formation of branching dendrites, whereas virtually all cells transfected with N17rac1 remained round or polygonal and failed to form dendrites in response to UV light (**Fig 7*c*, *d***; c-myc epitope).

Rac1 is localized to dendrite tips after treatment with NDP-MSH and UV light Rac1 is present in the cytosol bound to rho-GDI, a guanine nucleotide dissociation inhibitor that preferentially binds to GDP-bound rac1 (Chuang *et al*, 1993). When rac1 is activated, it dissociates from rho-GDI (Quinn *et al*, 1993; Bokoch *et al*, 1994) and is translocated to the plasma membrane where it regulates actin assembly (Ridley *et al*, 1992). Similarly, rac2, which is necessary for activation of the NADPH oxides system (Abo *et al*, 1991; Knaus *et al*, 1991, 1993), is translocated to the plasma membrane of neutrophils upon activation (Quinn *et al*, 1993), suggesting that localization of rac to membranes may be associated with its biologic functions of actin assembly and activation of oxidase enzymes. To determine if treatment of B16F1 cells with NDP-MSH or irradiation with UV light induced localization of rac1 to dendritic cell processes, cells were treated with NDP-MSH (10^{-8} M) for 24 h or were irradiated with a single dose

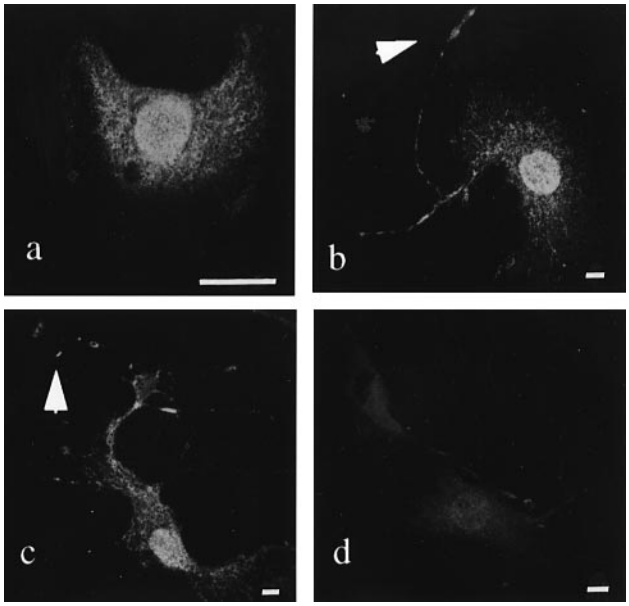

Figure 8. NDP-MSH and UV light induce the localization of rac1 to dendritic tips. B16F1 cells were either untreated (*a*), were treated with NDP-MSH (*b*) for 24 h (10^{-8} M), or were irradiated with a single dose of UV light from a solar simulator (*c*; 4.0 J per cm^2) and rac1 localization was detected by immunofluorescence staining and slides examined by confocal microscopy. Negative controls consisted of cells stained with rac1 antibodies preabsorbed with rac1 peptide (*d*). Rac1 is primarily located in the cell cytoplasm in untreated cells (*a*). NDP-MSH treated and UV irradiated cells exhibit dendritic cell processes in which rac1 is localized to dendrite tips (*arrowheads*; *b*, *c*). *Scale bar*: 7 μm.

(4.0 J per cm^2) of UV light from a solar simulator and 24 h later cells were stained with anti-rac1 antibodies and examined by confocal microscopy in order to localize rac1 in fine dendritic tips. Negative controls consisted of sections incubated with antibodies that were preabsorbed with rac1 peptides. Confirmation that rac1 antibodies had been completely preabsorbed was performed by western blotting with absorbed antibodies against rac1 peptides (not shown).

Untreated cells were round or polygonal and grew as small groups (**Fig 8*a***). In these cells, rac1 was present in the cytoplasm, predominantly in the perinuclear area. After treatment of B16F1 cells with NDP-MSH for 24 h, rac1 was localized to the tips of dendrites, although rac1 could still be visualized in the cell cytoplasm (**Fig 8*b***). After removal of NDP-MSH, and staining of cells 24 h later, cells had reverted to a round and polygonal morphology, and rac1 was present predominantly in the cytosol, unassociated with the plasma membrane (not shown). Twenty-four hours after a single dose of UV light, B16F1 cells became dendritic, similar to the morphology of NDP-MSH treated cells, and rac1 localized to dendritic tips (**Fig 8*c***). Twenty-four hours later, cells had reverted to a nondendritic phenotype and rac1 was localized to the cytoplasm (not shown). B16F1 cells stained with preabsorbed rac1 antibodies are shown in **Fig 8(*d*)**.

DISCUSSION

The melanocyte dendrite is a specialized structure for transport of melanosomes to neighboring keratinocytes whose formation is induced by hormones and UV light. The importance of actin as the infrastructure for melanosome transport has been highlighted by reports showing that melanosome transport to dendrites is absent in mice deficient in myosin V, an actin binding myosin motor, and ours and others results showing that actin and melanosomes co-localize in melanocyte and melanoma cell dendrites (Mercer *et al*, 1991; Provance *et al*, 1996; Wu *et al*, 1997). Whereas numerous reports have described a variety of hormonal and nonhormonal factors that induce melanocyte dendricity, the results presented in this report identify a molecular mediator of dendrite formation in a melanoma cell line. We showed that transfection of an expression vector encoding a constitutively active rac1 protein induced the formation of branching actin based structures in B16F1 murine melanoma cells and in four human melanoma cell lines. We chose to use B16F1 cells as a model system because of their ease of transfection compared with normal human melanocytes, and because their morphologic response to MSH and UV light is similar to normal human melanocytes (Lacour *et al*, 1992; Abdel-Malek *et al*, 1994). The morphology of V12rac1-expressing cells was distinctly different from cells expressing activated cdc42 and rhoA, which induced the formation of filopodia and stress fibers, respectively. Activated rac1 induced the formation of dendrite-like structures as early as 18 h after transfection with V12rac1 expression vectors, and dendrites persisted up to 3 d following transient transfection. We showed that the rac1-induced dendrites contained melanosomes, suggesting that coupling of actin to melanosomes is functional. Finally, we investigated the role of rac1 in mediating the well-known ability of α-MSH and UV light to induce dendrites in melanocytes and melanoma cells. Treatment of B16F1 cells with NDP-MSH or irradiation of these cells with a single dose of UV light from a solar simulator resulted in localization of rac1 to the tips of newly formed dendrites, suggesting that rac1 activation and dendrite formation are linked. The effect of NDP-MSH on rac1 distribution was reversible within 24 h after the removal of NDP-MSH from the medium, and 48 h after a single dose of UV irradiation, and corresponded with morphologic reversion to a nondendritic phenotype. The ability of a dominant negative inhibitor of rac1 to block both NDP-MSH and UV light induced dendrite formation shows that rac1 mediates the ability of these agents to induce dendrites in this model system. This was in contrast with a dominant negative inhibitor of cdc42 that failed to prevent the induction of dendrites in NDP-MSH-treated and UV irradiated B16F1 cells. Cells expressing the inactive rac1 protein, A35rac1, responded to NDP-MSH and UV light with the formation of dendrites, suggesting that the effect of N17rac1 in blocking dendrite formation was not a nonspecific effect of the vector.

The ability of a variety of different classes of hormones to activate rac1 suggests that it acts as a common downstream signaling intermediate for actin reorganization and shape change in response to multiple second messenger systems. Binding of α-MSH to its receptor activates adenylate cyclase in B16F1 cells, and some reports suggest that MSH activates the PKC signaling pathway, and increases intracellular Ca^{2+} levels in B16F1 cells (Mac Neil *et al*, 1990; Buffey *et al*, 1992). Presumably, these and other signaling pathways activate rac1 through the regulation of associated rac1 proteins such as rac1 GAP, rac1 GDI, or rac1 guanine nucleotide exchange factor. Potential candidate regulatory factors include vav, a guanine nucleotide exchange factor for rac1, cdc42, and rhoA (Han *et al*, 1997). Vav is activated by Lck kinase and Sck kinase (Teramoto *et al*, 1997), nonreceptor tyrosine kinases involved in the T cell receptor (Lck and Sck) and interleukin-15 (Lck) signaling cascade (Berg and Ostergaard, 1997; Adunyah *et al*, 1997). Ras-guanine nucleotide releasing factor (ras-GRF), a guanine nucleotide exchange factor for ras, is activated by G-protein coupled receptors and intracellular Ca^{2+}, suggesting a link between G-coupled receptor signaling, Ca^{2+} and ras activation (Mattingly and MaCara, 1996; Freshney *et al*, 1997). The signaling pathways activated in melanocytic cells in response to UV irradiation are just beginning to be defined (Im *et al*, 1998). While some reports showed that UV irradiation of pure cultures of melanocytes or S91 Cloudman melanoma cells does not increase cAMP levels (Friedmann and Gilchrest, 1987), more recent reports show that irradiation of human melanocytes results in a delayed (24 h post-irradiation) increase in cAMP levels to 80% above control levels (Im *et al*, 1998). These results suggest that PKA activation may be an upstream signaling pathway for UV-induced rac1 activation leading to dendrite formation. Others have shown that UVB irradiation activates guanylyl cyclase in human melanocytes (Romero-Graillet *et al*, 1996) and UVB and UVC irradiation acutely activates PKC in epithelial cells and fibroblasts (Matsui and DeLeo, 1990; Matsui *et al*, 1994, 1996). UV irradiation increases PKC mRNA and protein levels in human epithelioid P3 cells in culture, suggesting that irradiation may result in sustained activation of PKC through increases in PKC protein levels (Peak *et al*, 1991). Others have shown that UVB irradiation of B16F1 cells results in increased protein levels of the δ

subunit of PKC (Oka *et al*, 1996). These studies suggest that the PKC pathway may be involved in activation of rac1 in UV light irradiated B16F1 cells. Finally, UV light has been reported to upregulate the activity of MSH receptors and the autocrine production of α-MSH by melanocytes and S91 Cloudman melanoma cells (Bologna *et al*, 1989; Chakraborty *et al*, 1991, 1996; Pawelek *et al*, 1992), suggesting that rac1 activation in response to UV light may be mediated by autocrine production of α-MSH and upregulation of α-MSH receptor sensitivity, which could potentially activate multiple second messenger cascades including PKA, PKC, and intracellular Ca^{2+} signaling pathways.

The outgrowth of melanocytes dendrites is similar to the process of neurite outgrowth, as demonstrated by Kozma *et al* (1997) who showed that growth cone formation in N1E-115 cells involved rac1-dependent lamellipodia formation resulting in a gradual extension of neurite processes. Unlike dendrite formation in B16F1 cells, however, which requires only rac1, they found that neurite extension required both rac1 and cdc42. Studies in transgenic mice engineered to express constitutively active rac1 by Luo *et al* (1996) showed that dendritic spines in Purkinje cells were increased in number, implicating rac1 in dendrite formation in neural cells *in vivo*. Rac1 has been shown to mediate nerve growth factor induced neurite outgrowth in PC12 cells and acetylcholine induced growth cone formation in neuroblastoma cells (Altun-Gultekin and Wagner, 1996; Kozma *et al*, 1997), similar to the role of rac1 in α-MSH induced dendrite formation in the B16F1 murine melanoma model. These studies suggest that dendrite formation in melanocytic cells, which are derived from the neural crest and share morphologic features and express a similar profile of receptors as neural cells, could be used as a model system for investigation into the molecular mediators of neurite extension.

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